Gene Expression Analysis of Loto-Type Toll Receptors in the Onychophoran

*Euperipatoides kanangrensis*

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Abstract

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The expression and role of Loto genes was previously investigated in arthropods and it was concluded that the morphogenetic functions of Loto genes were conserved in the last common ancestors of arthropods. In this paper, possible orthologs of Loto clade genes are analysed and it was found that they possibly play a conserved role in convergent extension of cells from the blastoderm stage to germ band extension stage. The differences and similarities in the number of and expression of Loto clade genes in arthropods and onychophorans are compared and analysed. Unlike in arthropods, the expression of Euperipatoides Loto is strongest in the head including part of the somewhat enigmatic frontal appendages (the onychophoran antennae). I also find a stripped segmental pattern of expression that is similar to that classic expression in transverse segmental stripes of Loto class genes as seen in arthropods. However, these stripes are only week and appear to be mesodermal rather than ectodermal, as in arthropods. These facts are discussed in regard to current hypothesis about the origin of segmentation in panarthropods.

Insects, crustaceans and chelicerates each possess a set of several Loto class genes which are also involved in convergent extension. But the myriapod Strigamia only appears to possess one such gene. I therefore investigated Loto genes in another distantly related myriapod, Glomeris. It appears to only have one Loto gene and this appears to be involved in convergent extension as well. Two Toll genes of Glomeris (Gm-567 and Gm-596) were incomplete and could according to phylogenetic analysis represent Loto genes. I therefore checked their expression and found that they are not expressed like Loto genes. A third potential Glomeris Loto gene (Gm-399) could not be isolated via PCR.

My data suggest a somewhat different role for onychophoran Loto than convergent extension, and that the ancestral set of panarthropod Loto genes may be one, rather than a set.

Keywords: Panarthropods, Loto genes, Convergent extension

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Populärvetenskaplig sammanfattning

Analys av genuttrycket hos Loto-typ receptorer i klomasken, *Euperipatoides kanangrensis*
Linushiya R. Lionel


Min data tyder på att klomaskars Loto-gener har en annan roll än konvergent förlängning och att panarthropods kanske bara hade en Loto-gene istället för ett set av gener.

**Nyckelord:** Panarthropods, Loto genes, Convergent extension

*Examensarbete E1 i geovetenskap, 1GV025, 30 hp*
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**1. Introduction**

The Loto (long Toll) clade genes encode members of the Toll-like receptor class of proteins. It has recently been discovered that Loto genes are important factors in embryogenesis in axis elongation in arthropods (Benton et al. 2016, Pare et al. 2014). The expression and function of these genes were examined in arthropods such as the fruit fly *Drosophila melanogaster*, the beetle *Tribolium castaneum* and the spider *Parasteatoda tepidariorum* (Benton et al. 2016, Pare et al. 2014). It was found that these genes are involved in cell intercalation mediated embryo elongation (convergent extension) in all arthropods and it was suggested that the role of *Loto* genes has been conserved since the last common ancestor of all arthropods (Benton et al. 2016). Since the expression and function of Loto genes are identified in all the clades of arthropods, it is attempted to investigate if the Loto clade genes are present and expressed in comparable patterns during ontogenetic development of the onychophoran *Euperipatoides kanangrensis* then suggesting a conserved function of Loto genes already in the last common ancestor of Arthropoda and Onychophora, and thus in Panarthropoda as a whole. Onychophorans are targeted for the analysis since they are likely the closest relatives of Arthropoda (Campbell et al. 2011). If none of the Loto clade genes is expressed in comparable expression patterns as seen in arthropods, i.e. in transverse segmental stripes in the segment addition zone, then the function of Loto-clade gene(s) is likely not conserved in onychophorans and would present a phylotypic feature of Arthropoda. And this may then very much be the case because convergent extension is not a mechanism used by onychophorans to form their germ band during ontogenesis. However, onychophoran segmentation is in many aspects crucially different from its closest relatives, the arthropods (Janssen and Budd. 2013, reviewed in Janssen 2017). The major gene investigated in this study is *Ek-Loto* as it reveals an intriguing result in the phylogenetic analysis. Since only one single Loto gene was found in the myriapod *Strigamia* (Chilopoda) (Benton et al. 2016), I also investigated a clear Loto gene, *Gm-Loto*, and the two potential (as the latter two genes are incomplete) Loto genes – *Gm-567, Gm-596* in another myriapod, the pill millipede *Glomeris marginata* (Diplopoda). This should reveal if the somewhat derived complement of Loto genes is myriapod-specific, or specific for *Strigamia* (or its kin).

**2. Background**

**2.1 Arthropods**

Arthropods are invertebrate animals that appeared over 500 million years ago (Grimaldi, 2010). They have a segmented body, bilateral symmetry, hard exoskeleton, jointed legs, and multiple pairs of segmented limbs. Millions of species are estimated and are present in almost all environments. The basic arthropod body structure has evolved into many forms and has been adapted for different
functions. Both the internal and external organs are formed by the fusion of their segments. Their bodies are supported by a hard exoskeleton made of chitin, which they shed to produce a new and larger one in order to grow (moulting). Though their exoskeleton is hard, it is softer and bendable at the joints which facilitate the movement in the same way, which also could be considered as one of the examples for the evolutionary traits of arthropods. They have a number of limbs with distinct shape and size which also specialized for special functions in different species.

The phylum Arthropoda includes pancrustaceans (hexapods + crustaceans), chelicerates, and myriapods. Various analyses and discoveries proved that arthropods are monophyletic as they share a common ancestor (Chen et al. 1995). There are disputes in grouping the subphyla with each other based on the similarities and differences of their traits. However, the most accepted phylogenetic relationship describes Myriapoda as a sister group to Pancrustacea (crustaceans & Hexapoda) forming the monophyletic group Mandibulata (Regier et al. 2010). And Chelicerata as sister group to Mandibulata. The tardigrades and onychophorans are the closest relatives of arthropods, the three of them together are called Panarthropoda.

2.2 Onychophorans

Onychophorans are velvet worms, representing terrestrial invertebrates sharing characteristics with arthropods and annelids. They have a long segmented body with stubby legs (lobopods). It was suggested that onychophorans and other early Cambrian lobopods were similar to arthropods, which led to the development of the term Panarthropoda (Budd, 1996) and the animals with jointed limbs and hardened cuticles were named as Euarthropoda (Nielsen, 2001). The onychophorans differ from arthropods by having non-jointed limbs. Although relatively little information is available on their biology and embryonic development, there are quite a few analyses revealing that they have a distinct developmental mode from other arthropods which involves different morphological and genetic factors (Janssen and Budd, 2013; Janssen et al., 2015).

2.3 Developmental modes of arthropods

The fruit fly Drosophila melanogaster represents the best-understood arthropod model system. The mode of embryogenesis employed by Drosophila is termed as long germ embryogenesis, which otherwise can be defined as the establishment of all segmental fates at the blastoderm stage prior to gastrulation (Davis & Patel, 2002). The establishment and patterning of anterior-posterior (AP) axis are mediated by the so called segmentation genes. The process of AP axis formation is initiated by the maternal coordinate genes that provide broad, graded information from both the ends of the embryo which is then interpreted by the gap genes (first zygotically activated segmentation genes). The expression domains of these genes subdivide the embryo into large, partially overlapping yet precisely defining blocks, and provide short distance graded information. The pair rule genes interpret these
gradients and are expressed in a double segment periodicity which initiates the metameric stage of the fly. The segment polarity genes interpret these expressed patterns and facilitate the establishment of borders between the segments and fates within each segment (Lynch & Desplan, 2010).

Insects of more basally branching lineages are employed by an alternative embryonic process called short germand intermediate germ embryogenesis based on the number of segments established at the blastoderm stage. In these embryogenesis processes, the most anterior segments establish earlier while the posterior segments are generated later progressively from a posterior region called segment addition zone (Brent et al., 2007) (Lynch & Desplan, 2010).

Almost all the other arthropods (all except Drosophila, their closest relatives, and some groups of beetles) undergo short term embryogenesis that follows two stages. For example, Oncopeltus (Hemiptera, true bugs) first stage is blastoderm stage – in which the six anterior segments (gnathal and thoracic segments) are formed simultaneously and the expression pattern of segment-polarity genes is reminiscent to that of Drosophila. Second is the germ band stage - in which the segments are added sequentially one by one from a posterior located segment addition zone (Stahi, 2016). There is an interference of the Notch/Delta signalling pathway in Oncopeltus during pair-rule gene function, which is unlike the situation in Drosophila. Replacement of an N/Dl signalling by a gap-gene system may have led to the transition from long to short germ embryogenesis (Rosenberg et al. 2014).

Similarly, in Gryllus (Orthoptera, grasshopper) only the head and thoracic segments are formed simultaneously and as the AP axis elongates, the abdominal segments are added sequentially.

In the spider Parasteatoda, the AP axis is established during germ-disc formation which is one of the most important steps in spider embryogenesis during which the centre of the germ-disc will become the posterior pole and the rim of the germ-disc will become the anterior pole (Pechmann, 2016). It is unclear whether the spider embryo formation involves in cellular rearrangement, cellular migration or combination of both. However, again only the most anterior segments form from the blastoderm and more posterior segments form from a posterior segment addition zone (Pechmann, 2016).

In the myriapod Strigamia, the embryogenesis incorporates both cellular migration and cellular division; early blastomeres divide at the centre of the egg and produces a huge population of cells which migrate peripherally to the surface of the egg, the blastoderm becomes multilayered nearby where the nuclei remain sparse (blastopore), this multilayer region extends from the blastopore towards the anterior region of the egg. The trunk and leg segments are then added sequentially (Brena & Akam, 2012). It is thus clear that most arthropods including all crustaceans, all myriapods, all chelicerates, and the very most of insects develop via a short-germ mode of development. The developmental mode represented by Drosophila, its closest relatives, and some other lines of insects is thus derived.
2.4 The developmental mode of onychophorans

Although onychophorans are closely related to arthropods, the development of onychophorans is crucially different from arthropods. Some of the arthropods undergo protostomic development in which the blastopore only develops into mouth while other arthropods undergo deuterostomic development (Anderson, 1966; Hejnol & Martindale. 2009). Onychophorans employ neither protostomous nor deuterostomous developmental modes but ‘concealed deuterostomy’. The ‘concealed deuterostomy’ is a convergent developmental mode. The protostomic and deuterostomic developmental modes are observed in some of the onychophorans whereas in others such as the velvet worm *Euperipatoides kanangrensis*, the blastopore develops into a single slit-like furrow along the anterior-posterior axis. The anterior part of the furrow forms into mouth while the anus forms posterior to mouth- anus furrow, after the median closing down (Janssen et al. 2015).

However, interestingly, all segments of onychophorans (head and trunk segments) form sequentially one by one from the posterior segment addition zone that is located on either side of the posterior pit which lies posterior to the anus (Janssen et al. 2015). From here, ectoderm as well as mesoderm derives. The latter is indicated by the expression of the conserved mesodermal marker gene *twist* (reviewed in Janssen 2017). So, unlike the situation in arthropods (or the extreme as seen in long germ arthropods), no segments form directly from the blastoderm stage. Furthermore, it is unclear if convergent extension is part of onychophoran development. In comparison to arthropods, there is a much smaller segment addition zone which is restricted to a very restricted posterior area. In arthropods, like e.g. *Strigamia*, the segment addition zone can be huge, especially at early developmental stages. This segment addition zone then shrinks during development and with the addition of more and more segments. And this process includes the mechanism of convergent extension (intercalation of cells).

2.5 Germ band formation via convergent extension

An embryo is able to generate its shape through morphogenesis which requires proper fate speciation and proper movement of cells without changing the number of cells (Walck-Shannon & Hardin. 2014). One of these cell movements is cell intercalation which lengthens or spreads a tissue at the expense of narrowing along its AP axis. One such cell intercalation movement is convergent extension, in which a tissue narrows along an axis and lengthens in another direction that elongates the anterior-posterior (AP) axis of an animal. Convergent extension has been studied in many animals such as fishes and frogs, which are ideal candidates due to their large embryo size. Different animal embryogenesis utilize convergent extension in different manner, either as cellular rearrangement or cellular migration (Wallingford et al. 2002). Cellular rearrangement is the process by which individual cells of a tissue rearrange to reshape the tissue as a whole, while cellular migration is the directed movement of a singular cell or small group of cells across a substrate such as a membrane or tissue. In
epithelial tissues, cell intercalation is mediated by spatially regulated actomyosin-contractility that induces oriented cell arrangements that then orchestrates elongation of tissues. This mechanism was, as so many others, first discovered in the model arthropod *Drosophila* (Fernandez-Gonzalez et al. 2009).

### 2.6 Toll genes

The toll genes encode members of the toll-like receptor class of proteins. They were first identified in *Drosophila melanogaster* (Hansson & Edfeldt, 2005) and are found to be very important in embryogenesis, in establishing the dorsal-ventral axis. There are thirteen Toll genes (roughly named as TLR1-TLR13) that have been identified in humans and mice together so far and in *Drosophila* *Tl*-2, *Tl*-6, *Tl*-7, *Tl*-8, *Tl*-10 have been discovered. Earlier, both the Toll and Long toll (Loto) genes together were called Toll genes. Later, when the structural difference between each gene was observed, certain members of the Toll class genes were categorized as Long Toll (Loto) genes by Benton and colleagues (2016). These genes are responsible for dorsal-ventral patterning in insects and are involved in the regulation of innate immunity in arthropods and vertebrates (*Tl*-2 and *Tl*-8) (Paré et al. 2014). The maternal Toll orthologs are required for establishing germ layers along the dorsal-ventral axis and for embryonic patterning along the anterior-posterior axis during embryogenesis in insects (Berni et al. 2014). Not only insects but also various species including vertebrates and invertebrates employ the role of Toll genes in embryonic development and pathogen recognition (Pujol et al. 2001). One of the significant evolutionary traits of the Toll like receptors (TLR) is that they conserve innate immune mechanism for recognizing specific pathogen molecules - for example: In fruit flies they enhance the susceptibility to fungal infections, and in mice they enhance susceptibility to Gram-negative bacterial infection (Beulter and Poltorak. 2000).

In arthropods, it was observed recently that the three genes *Tl*-2, *Tl*-6 and *Tl*-8 are required for cell intercalation during axis elongation and neither of these receptors can fully substitute for each other functionally, particularly *Tl*-2 which is necessary in orienting edge formation (Paré et al. 2014).

### 2.7 Long toll genes (Loto) and their role in convergent extension

Certain members of toll genes such as *Tl*-6, *Tl*-7, *Tl*-8 and *Tl*-10 are “longer” and encode more leucine rich repeats (LRR) as well as a conserved cytoplasmic Toll/Interleukin-1 receptor (TIR) domain (Benton et al. 2016, Paré et al. 2014). Recent studies have shown that these specific Toll genes also play a conserved role in arthropod convergent extension. Therefore, Toll genes with these characteristics, a high number of LRRs and a TIR domain, have been called Long Toll or Loto genes (Benton et al. 2016). For example, *Drosophila* *Tl*-8 which encodes a single-pass transmembrane protein contains 27 extra-cellular LRRs and a conserved TIR domain, and is involved in convergent extension (Paré et al. 2014).
2014). The Loto genes are involved in the activity of controlled intercalation of cells between their neighbours during the development of arthropod embryos (Benton et al. 2016).

Various other studies also revealed that the Loto clade genes are not only directing axis elongation but are also expressed and involved in the formation of the embryonic head, appendages, and the mesoderm. They are also involved in the development of ovary, leg and wing, salivary glands and nervous system (e.g. Benton et al. 2016).

2.8 Loto genes in *Drosophila*

In *Drosophila*, the members of the Loto gene family are expressed in overlapping stripes along the AP axis which is due to the activity of Even-skipped and Runt transcription factors since they direct cell behaviour during convergent extension (Paré et al, 2014). It was analysed that *Tl2, Tl6, Tl7* and *Tl8* are very much needed for plane polarity, cell intercalation and convergent extension in *Drosophila*. Of them *Tl6, Tl7* and *Tl8* belong to the Loto clade genes. *Tl7* being a member of Loto clade genes, is also involved in convergent extension but is present at low levels. Paré and his colleagues (2014) were investigating the role of Toll and Loto genes by disrupting the respective genes in mutant embryos (Eve and Runt mutants, since those transcriptional factors control cell behaviour). Axis elongation was found to be reduced by 20% in the embryos defective for *Tl2* and *Tl6*, nearly 40% in embryos defective for *Tl2, Tl6* and *Tl8*. Similarly the embryos defective for *Tl2, Tl6, Tl8 eve* and *runt* mutant embryos (simultaneously disrupted) showed reduced planar polarity, rapid edge contraction and edge formation. Thus it indicates that *Tl2* of Toll gene family and *Tl6, Tl8* of Loto clade gene family act together to direct cell intercalation.

2.9 Loto clade gene expression in other arthropods

2.9.1 *Tribolium*

In the beetle (Hexapoda) *Tribolium castaneum*, the Loto genes are involved in convergent extension of the embryo starting at the stage when the germ band forms and continuing throughout germ band elongation. *Tl6, Tl7, Tl8* and *Tl10* of Loto clade gene family that have a role in cell intercalation in *Drosophila* and all of those genes were expressed in stripes in *Tribolium* during segmentation which is a similar pattern as described in *Drosophila* (the latter has lost *Tl10*). The primary domains are expressed in the blastoderm stage and the secondary domains appeared in every segment at the matured stage (germ band extension stage) reminiscent to *Drosophila* (Benton et al. 2016).

2.9.2 *Nasonia*

In the wasp (Hexapoda:Hymenoptera) *Nasonia vitripennis*, Nv-*Tl7*and Nv-*Tl10* are expressed in stripes and their domains arise in an anterior-to-posterior progression similar to the *Tribolium*
expression pattern. The expression starts at the early blastoderm stage and persists until the extended germ band stage. For both genes, the earliest expression is in the dorsal presumptive extra-embryonic tissue. Although the posterior \textit{Nv-Tl7} domains arise earlier than \textit{Nv-Tl10} domains, segmentation appears in an anterior-posterior progression (Benton et al. 2016).

**2.9.3 Oncopeltus and Gryllus**

In the milkweed bug (Hexapoda:Hemiptera) \textit{Oncopeltus fasciatus}, \textit{Tl7}, \textit{Tl8} and \textit{Tl10} are expressed in stripes from early blastoderm to extended germ band stages with a noticeable early expression of \textit{Tl10} and later expression of \textit{Tl7} in the presumptive serosa. The same orthologs are expressed in the cricket (Hexapoda:Orthoptera) \textit{Gryllus bimaculatus}, in stripes throughout germ band development stages (Benton et al. 2016).

**2.9.4 Parhyale**

In the crustacean \textit{Parhyale hawaiensis}, \textit{Ph-LotoA} is expressed in odd-numbered parasegments. It is strongly expressed in the posterior segment which exhibit in a single stripe and weakly expressed in the matured anterior segments unlike other \textit{Toll} genes (Benton et al. 2016). It is also expressed from blastoderm to extended germ band stages.

**2.9.5 Strigamia**

In the centipede (Myriapoda) \textit{Strigamia maritima}, \textit{Sm-LotoA} is expressed in an impressive striped pattern in the posterior, medial and anterior regions. Expression with double-segmented periodicity arises in the posterior region and persists through the matured segments, leading to a neural-like expression pattern in the anterior segments of the embryo (Benton et al. 2016).

**2.9.6 Parasteatoda**

In the spider (Chelicerata) \textit{Parasteatoda tepidariorum}, \textit{Pt-LotoA} and \textit{Pt-LotoB} are expressed during embryo formation. \textit{Pt-LotoA} is expressed in the cheliceral segment and the second to fourth walking-leg segments and domains are expressed sequentially during the formation of opisthosoma in a striped pattern and fades as the germ band elongates while new domains arise in the head, and the cheliceral segment. \textit{Pt-LotoB} is expressed initially in anterior domain and in the forming caudal lobe and then fades while new expression arises in the maturing head. During germ band formation, a new domain arises in the third walking-leg bearing segment but fades as the embryo matures. The expression in the caudal lobe persists throughout germ band extension (Benton et al. 2016).

**3. Materials and Methods**

**3.1 RT-PCR and gene cloning**

The previously obtained cDNAs from the mRNAs of \textit{Euperipatoides kanangrensis} and \textit{Glomeris marginata} by reverse transcription were provided by Dr. Janssen. These cDNAs were served as
templates for PCR to isolate *Ek-Loto* from *Euperipatoides kanangrensis* and *Gm-Loto*, *Gm-567*, *Gm-596*, and *Gm-399* from *Glomeris marginata*.

The respective primer sets fw1/bw1 and fw2/bw2 (for nested PCR) were used to isolate *Gm-Loto*, *Gm-567*, and *Gm-596* whereas *Gm-399* could not be isolated which might be due to the absence of the gene in cDNA or contamination of the available transcriptome data (for primer sequence, see Table 1). The fragments of isolated genes were obtained from an agarose gel in order to avoid unwanted side-products (Gen Elute Gel Extraction Kit/Sigma).

**Table 1. Primers used to amplify gene fragments.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EkLoto-fw1</td>
<td>CTGCCCAAAATTGTAGACCTG</td>
</tr>
<tr>
<td>EkLoto-bw1</td>
<td>CGTCTTGATCCTTTGGCTTAG</td>
</tr>
<tr>
<td>GmLoto-fw1</td>
<td>CCAACAACTCGCTGTCGTCGC</td>
</tr>
<tr>
<td>GmLoto-bw2</td>
<td>GACAGACGCTTGGGTGAGCGC</td>
</tr>
<tr>
<td>GmLoto-bw1</td>
<td>AAGCCCTATCTTCAGCAACCG</td>
</tr>
<tr>
<td>GmLoto-bw2</td>
<td>GCTGAGGAGGACGAGATGAGG</td>
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<td>Gm596-fw1</td>
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<td>Gm567-bw2</td>
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<tr>
<td>Gm399-fw1</td>
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<td>Gm399-fw2</td>
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<tr>
<td>Gm399-bw1</td>
<td>ACTGGATAGGTGTTCGAAAA</td>
</tr>
<tr>
<td>Gm399-bw2</td>
<td>CATGAAAGGGACATTACTCAA</td>
</tr>
</tbody>
</table>

The gene fragments were cloned by ligating into bacterial vectors using a TOPO cloning kit and transformed with chemically competent *E.coli* cells. These cells were then grown on petri dishes with X-gal and kanamycin for overnight at 37°C. A Colony PCR was performed to identify the plasmids with right-sized templates. The plasmids with expected sized templates were extracted by using Sigma GenElute Plasmid Mini prep kit. The extracted plasmids were checked for the concentration and purity using a photometer. The clones were sequenced and blasted (blastx) to determine the direction of the sequence in order to synthesize probes. Template PCR (with M13F/M13R) was performed to linearize the templates for further probe synthesis.

Template PCR products were purified by QIA-Quick PCR purification kit.
3.2 Probe synthesis
DIG-labelled anti sense RNA probes were synthesized from template PCR products at 37°C for four hours (for protocol, see Appendix 1). The probes were then cleaned by using the Qiagen RNeasy Mini Kit.

3.3 Whole-mount in situ hybridization
The embryos of *Euperipatoides kanangrensis* and *Glomeris marginata* were provided by Dr. Janssen. Vitelline membranes of fixed embryos of *Glomeris* were removed manually using forceps under a stereo- microscope and stored in methanol. The whole-mount in situ hybridization protocol was followed for hybridizing the probes of the onychophoran and the myriapod with their respective embryos (Appendix 1).

3.4 Image taking
The images of all the hybridized embryos were taken under a Leica microscope equipped with a Leica DC 100 digital camera.

3.5 Sequence analysis
The complete protein sequence (as far as present) of the putative Loto genes from *Euperipatoides* and *Glomeris* were aligned with the identified Loto and Toll genes from various arthropods using ClustalX with default parameters in MacVector v12.6.0.

A Bayesian phylogenetic analyses was performed with MrBayes (Huelsenbeck and Ronquist 2001) using a fixed WAG amino acid substitution model with gamma-distributed rate variation across sites (with four rate categories). An unconstrained exponential prior probability distribution on branch lengths and an exponential prior for the gamma shape parameter for among-site rate variation was applied. Tree topology was calculated by using 1,100,000 cycles for the MCMCMC (metropolis-coupled Markov chain Monte Carlo) analysis with four chains and the chain-heating temperature set to 0.2. The Markov chain was sampled every 200 cycles. Clade support was assessed with posterior probabilities computed with MrBayes.

The cDNA sequences were blasted (blastx) and the TIR count was checked by using the NCBI link (Table 2).

The number of LRRs of each gene of this study was obtained by using the LRR search tool (Table 2).

The DNA sequences were translated into amino acid (protein) sequence in order to count LRR by using the online tool.
Table 2. Summary of LRR and TIR domains in published and new Toll/Loto genes

<table>
<thead>
<tr>
<th>Animal</th>
<th>Gene</th>
<th>Number of LRR</th>
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4. Results

4.1 Phylogenetic analysis

The phylogenetic tree helps to determine if the genes of interest of this study (Ek-Loto, Gm-Loto) fall under either Loto or non-Loto clade genes. The position of Ek-Loto in the phylogenetic tree is very intriguing as it falls between the Loto and non-Loto genes (Fig. 1), even though it has a structure of a typical Loto gene such as encoding a large number of LRRs and 2 TIRs (Table 2). Hence Ek-Loto expression pattern is analyzed in this study in order to investigate if the respective gene conserves Loto gene characteristics.

The phylogenetic analysis indicates clearly that Gm-Loto falls under Loto clade genes as it branches together with Strigamia maritima Sm-LotoA which means Gm-Loto closely resembles Sm-LotoA. This fact is supported by the observed expression pattern of Gm-Loto which is explained in the following section. Gm-567 and Gm-596 both fall between the Loto and non-Loto genes (Supplementary Fig. S1), it cannot be considered as valid since they are incomplete. They could
possibly represent Loto genes or Toll genes. I therefore decided to investigate the embryonic expression patterns of these genes as well.

The *Glomeris* gene *Gm-399* (another incomplete Toll gene sequence with the potential of being a Loto gene) clusters with the putative *Euperipatoides* Loto gene *Ek-Loto* which makes it a very interesting gene for comparative studies. Unfortunately, it was not possible to isolate this gene from the provided cDNAs.

![Figure 1. Phylogenetic analysis. Green branches represent Loto clade genes. Red branches represent non-Loto genes. Blue branch labels *Ek-Loto* that falls between Loto and non-Loto genes. Abbreviations: *Dm*, *Drosophila melanogaster*; *Tc*, *Tribolium castaneum*; *Sm*, *Strigamia maritima*; *Gm*, *Glomeris marginata*; *Tl*, *Toll*; *Loto*, *Long Toll*.](image-url)
4.2 Expression of *Euperipatoides kanangrensis* Loto (Ek-Loto)

*Figure 2. Expression of Ek-Loto.* In all the panels anterior is to left. (A) Ventral view. Stage 9 embryo showing expression in the developing segments (arrow heads). (A1) DAPI of (A). (B) Ventral view. Expression at the posterior emerging segments, the saz (asterisks), and in the forming frontal appendages (arrows). (C) Dorsal view. Expression in the segments (arrow heads). (D) Lateral view of (C). Expression in the segments (arrow head). (D1) DAPI of (D). (E) Ventral view. Expression in posterior segments (arrow heads). Expression in the rear end of anal valve. (F) Ventral view. Stage 13 embryo showing expression in the developing posterior pit (asterisk) same as (E), and in the frontal appendages (arrow). Abbreviations: a, anus; fap, frontal appendage; hl, head lobe; j, jaw bearing segment; L, walking limb; m, mouth; pp, posterior pit; saz, segment addition zone j; sp, slime papilla; st, stage; DAPI, 4’,6-diamidino-2-phenylindole (fluorescent stain).

*Euperipatoides kanangrensis* Loto (Ek-Loto) is first expressed weakly in form of transverse segmental stripes in all segments (Fig. 2a, and not shown). Embryos are showing expression of *Ek-Loto* from the early germ band to extended stages. From approximately stage 11 on, *Ek-Loto* is expressed in the dorsal region of the head that also partially covers the primordia of the now outgrowing frontal appendages. At this stage, the expression is also clear and strongest in the newly forming segments in the segment addition zone on either side of the posterior pit region (Fig. 2B). This general expression persists in later stage embryos (Fig 2C-F). Then, in even later stage embryos, expression appears along the dorsal side of limb-bearing segments and inside each limb bud (Fig. 3 A). The initial expression in the head lobes is now also present in the complete posterior and dorsal region of the head lobes (Fig. 3B). Note that in figure 3-B, the expression pattern in the trunk is not visible which is likely due to improper embryo fixation.
Figure 3. Expression of *Ek-Loto*. In all the panels anterior is to left. (A) Lateral view. Stage 15 embryo showing expression in the frontal appendages and the posterior of the head lobe (arrow). Asterisks show the expression in the segments. Arrow heads point to the expression along the dorsal rim to the trunk segments. Arrow marks expression in the head lobes. Closed circles indicate expression in the walking limbs. (A1) shows DAPI of (A). (B) Lateral view. Stage 13 embryo showing expression in the head lobes; note that expression in the trunk is very low due to poor fixation of the embryo in this region. (B1) DAPI of (B). Abbreviations as in Fig.2.
4.3 Expression of *Glomeris marginata* *Loto* (*Gm-Loto*)

*Gm-Loto* appears to be expressed from the stages in which segmentation starts. Initially *Gm-Loto* is expressed in the forming segment addition zone (Fig. 4A). Then transverse stripes of expression appear in the mandibular, maxillary, postmaxillary and the first trunk segment (Fig 4B). *Gm-Loto* is also expressed in the head lobes and ubiquitously at weak levels (Fig. 4B-E). As the germ band extends, a strong expression in the antennal segment, the labrum, the ventral trunk segments and limbs is visible (Fig. 4C-E). From stage 3 onwards the dorsal ridge of the trunk segments also shows a strong expression (Fig.4 C-F). *Gm-Loto* is transiently expressed in form of transverse stripes in newly forming posterior segments (Fig. 4C-E). These stripes then transform into patch-like segmental domains which likely represent expression in the ventral nervous system (Fig. 4C-E). A clearly visible strong expression is observed in the tergite boundaries at late developmental stages (Fig. 4 F).
4.4 Expression of *Glomeris marginata Gm-567* and *Gm-596*

Both genes, *Glomeris Gm-567* and *Gm-596*, which represent putative Loto class genes (see discussion for further information) are expressed ubiquitously at all investigated developmental stages (Fig. 5).

![Expression of Gm-567 and Gm-596](image)

Figure 5. Expression of *Gm-567* (A,B) and *Gm-596* (C,D). All embryos oriented with anterior to the left. (A,B,C,D) Ubiquitous expression in all the embryonic stages. Abbreviations as in Fig.4.

5. Discussion

5.1 Identification of Loto class genes

I found one clear Loto class gene in *Glomeris* (*Gm-Loto*). Two genes clustered at the root of Loto genes (*Gm-567* and *Gm-596*). Both genes are incomplete and it is therefore impossible to say, how the overall gene structure is; i.e. how many LRR domains and TIR domains they possess. This however, and especially the number of LRR domains is crucial for the identification of Loto genes and to distinguish them from other Toll genes. However, since these genes are not expressed in Loto-like expression patterns, I assume that they do not belong to Loto class of genes. That leaves one Loto gene for *Glomeris*. This is the same as in another myriapod, *Strigamia*, suggesting that there is only one
Loto gene in myriapods. Two Loto genes have been described for the spider *Parasteatoda*, but this number may be due to a whole genome duplication in this branch (Schwager et al. 2017), suggesting that one Loto gene was present in the last common ancestor of all arthropods. Since I only find one Loto gene in the onychophoran, this assumption is further supported.

5.2 Expression in stripes: indicator for conserved function?

Typical for Loto genes that are involved in convergent extension is their expression in form of stripes, either in the blastoderm stage (for long-germ arthropods like *Nasonia* and *Drosophila*), or in the segment addition zone and newly formed segments as in short-germ arthropods like *Tribolium* or * Gryllus*. In onychophorans, none of the segments is formed directly from the blastoderm stage, but all segments come from the posterior located segment addition zone. I find very weak expression in stripes in all formed segments. These stripes persist relatively long, and do not like in most arthropods disappear or transform into other patterns. Also, given that the expression signal that I detect with in-situ hybridization is very weak it is possible that the expression is mesodermal rather than ectodermal as in arthropods. It is thus unclear if the stripe pattern that is found for *Ek-Loto* in *Euperipatoides* is correlated with convergent extension as it is in arthropods. The function of *Ek-Loto* in the onychophoran could be different, but related to that of Loto genes in arthropods. The Loto gene could have been recruited for convergent extension in arthropods, but may be involved in mesoderm patterning in onychophorans. Or it could be a sign for mesodermal segmentation (discussed below).

Anyhow, the expression of the *Glomeris* Loto gene (*Gm-Loto*) is clearly conserved and may likely have a function in convergent extension. It is expressed in stripes similar to what is seen in other arthropods. And also other aspects of its expression are similar to that seen in other arthropods (and also the onychophoran (discussed below). Interestingly, the stripy pattern of Gm-Loto is much less clear and impressive as that of the single Loto gene of another myriapod, *Strigamia*. *Sm-Loto* is expressed in many more stripes and much broader stripes. This can be correlated to the form of the segment addition zone. In *Strigamia*, many more segments form from this zone, and thus, this zone is much broader. In *Glomeris*, only a few segments form during embryogenesis from the segment addition zone, and indeed the stripes of *Gm-Loto* are much fewer and less prominent.

5.3 Mesodermal vs ectodermal segmentation in arthropods and onychophorans

*Ek-Loto* is expressed in all the trunk segments as transversal stripes reminiscent to arthropod (e.g. *Drosophila*) expression pattern but it is not strongly visible. This may be due to the mesodermal segmentation of onychophoran. Arthropods are employed by ectodermal segmentation in which the segmentation is controlled by ectoderm, which then later spread the signals to mesoderm causing mesoderm segmentation (Hannibal et al. 2012; Bock, 1941) whereas onychophorans represent
mesodermal segmentation and the metameric formation of somites as the clearest ontogenetic sign of that (mesodermal segment blocks) (Janssen and Budd. 2013, 2016). The striped pattern of Ek-Loto is thus not directly to compare with the stripes in arthropods. In Euperipatoides the stripes are likely mesodermal, in arthropods they are ectodermal. If the function is related, however, then it could represent a switch from mesodermal segmentation as in onychophorans to ectodermal segmentation as in arthropods. Although convergent extension is not a direct segmentation mechanism it is at least regulated (in at least insects) by pair rule genes (segmentation genes) (Benton et al. 2016, Pare et al. 2014). It is thus a connected and related mechanism, convergent extension and segmentation go hand in hand in arthropods.

Some authors believe that mesoderm segmentation is ancestral and that ectodermal segmentation of arthropods is a derived feature and an adaptation of the arthropods (Budd 2001). It is therefore interesting to find a segmentation-connected gene (Ek-Loto) to be expressed in segmental patterns in the mesoderm. There could be a connection.

5.4 On the homology of the arthropod labrum and the onychophoran frontal appendages

The arthropod labrum and its origin (homology to other structures?) is discussed a lot (e.g. Janssen 2017, Ortega-Hernandez et al. 2017, Posnien et al. 2009). One theory claims that the labrum is homologous to the frontal appendages of onychophorans. This is supported by Hox gene expression patterns (Eriksson et al. 2010, Janssen et al., 2014) and partially by the expression of limb-patterning genes and anterior marker genes (Janssen 2017). One such anterior marker is Six3 that is expressed in the labrum and the frontal appendage. Since this discovery (Steinmetz et al. 2010) researchers look for more specific markers to possibly homologize labrum and frontal appendage (Janssen 2017).

I found that the Ek-Loto gene is expressed strongly in the onychophoran head and also in half of the frontal appendages. Interestingly I also found a closely related gene in the myriapod Glomeris (Fig. 1). This gene, Gm-c399, could be expressed in very similar pattern as Ek-Loto and thus could be expressed more or less exclusively in the labrum. Very unfortunately, however, I could not isolate this gene because the PCR did not work. This could be because it is not represented in the cDNAs I had, or because it is a transcriptome data-contamination and is not a Glomeris gene at all. Or it is expressed at very low levels. If it is a real gene from Glomeris then it would be very interesting to try again to isolate it.

5.5 Conserved and diverged patterns of Loto genes

Some aspects of Loto gene expression beyond the described pattern in transverse segmental stripes are conserved in arthropods. For example expression in the ventral nervous system and the head (brain). This is seen in insects, the spider and myriapods, including Glomeris (Fig. 4). Loto genes appear also
to have a function in the limbs where they are expressed too. In *Glomeris* and the spider *Parasteatoda* (*Pt-LotoA* gene) Loto is expressed in the dorsal tissue of the trunk/opisthosoma where the heart may form. This is also the case for the onychophoran (Figs 2 and 3), but not for another myriapod, *Strigamia* (as one can see from the data in Benton et al. 2016). Unlike the situation in arthropods, the onychophoran Loto gene is not active in the ventral nervous system. The domain in the head is likely different from that in the head/brain of arthropods.

6. Conclusions

From the results, there are no adequate evidences to prove that *Ek-Loto* like gene conserve all the Loto gene characteristics instead it only shows partial conserved characteristics. *Gm-Loto* is undoubtedly a Long toll gene as it falls within the Loto family in the phylogenetic analysis, also it reveals similar expression pattern reminiscent to other arthropods and the myriapod *Strigamia* (centipede) in particular. *Gm-567* and *Gm-596* are not Long toll genes as they are ubiquitously expressed.

7. Acknowledgements

I thank Dr. Ralf Janssen who is the supervisor and project-head for guiding me with laboratory experimental procedures and interpreting the outcome of experiments and Prof. Dr. Graham E. Budd for project fund. I also want to mention that Dr. Janssen was mainly responsible for sequence and phylogenetic analysis.

This work has been supported by the Swedish Research Council. I gratefully acknowledge the support of the NSW Government Department of Environment and Climate Change by provision of a permit SL100159 to collect onychophorans at Kanangra Boyd National Park, and to the Australian Government Department of the Environment, Water, Heritage and the Arts for export permits WT2009-4598 and WT2012-4704.
8. References


**Internet resources:**


Appendix 1 Protocols

**Probe synthesis**

In 1.5 ml tube

3 μl H2O

3 μl PCR product [template]

1 μl transcription buffer 10x

1 μl RNase OUT (inhibitor)

1 μl DIG rNTP mix

1 μl RNA-polymerase (T7, T3 or Sp6 in this project)

Incubate at 37°C for 2-4 hours

**Whole-mount in situ hybridization**

Post Fixation and ProteinaseK digestion

- Return embryos to RT
- Wash 5 min in 50% MeOH in PBS-T
- Wash 5 min in 30% MeOH in PBS-T
- Wash three times in PBS-T for 10 min each
- Fix for 20 min in 5% FA in PBS-T (1 ml PBS-T + 170 μl 37% FA)
- Wash three times in PBS-T for 5 min each
- Incubate with ProteinaseK (3.5 μl in 10 ml PBS-T)
  
  *Euperipatoides*: 4 min @ RT
  
  Rinse briefly with PBS-T
- Wash twice in PBS-T for 5 min each
- Fix for 20 min in 5% FA in PBS-T (1 ml PBS-T + 170 μl 37% FA)
- Wash three times in PBS-T for 5 min each

Prehybridization

- Wash embryos once in 1:1 PBS-T:HYB-B for 15 min (first add 500μl PBST, then 500μl HYB-B (mix gently by inverting)
- Replace and incubate embryos in 500 μl HYB-B for 5 min @ 65°C
- Replace HYB-B with 500 μl of pre-warmed HYB-A
- Pre-hybridize @ 65°C for 2h in HYB-A
- Heat probes to 95°C for 2 min and place on ice for 5 min
- Preheat probes to 65°C
Hybridization

- Remove HYB-A from the embryos without letting them touch air
- Add 50 µl of pre-warmed fresh HYB-A
- Add 3 µL probe, mix gently, and incubate overnight @ 65°C

Probe removal

- Wash two times with 500 µl pre-warmed HYB-B, incubate 20 min @ 65°C
- Replace with 1:1 mixture of HYB-B:2XSSC incubate 30 min @ 65°C
- Wash four times with 2xSSC for 20 min each @ 65°C
- Wash four times with PBS-T for 10 min each @ RT
- Incubate in blocking buffer for at least 2 h

Detection

- Prepare 1:2000 dilution of AP conjugated DIG-antibody in 1 ml blocking buffer
- Incubate embryos with DIG-antibody mixture for at least 1.5 h
- Wash four times in PBS-T for 10 min each
- Incubate in PBS-T overnight @ 4°C

Staining

- Wash three times with PBS-T for 5 min
- Wash two times with AP staining buffer (pH 7.4) for 10 min
- Incubate with BM purple staining solution and monitor color development under the microscope (stain in the dark, staining @ 4°C will take longer, but will also enhance signal to noise ratio)
- Stop staining reaction by washing at least 5 times with Stop solution for 10 min each
- Counterstain with 2 µl/ml DAPI for 30 min (in the dark)
- Remove solution by washing in Stop solution min. 3x10 min
- For storage, add 50-100 µl 37% FA to 1 ml Stop solution and store @ 4°C

**10x PBS stock**

1.37 M NaCl
27 mM KCl
100 mM Na2HPO4
adjust pH to 7.4
autoclave

**PBS-T**

1 x PBS
0.02 % Tween-20
**HYB-B**
50% formamide
25% 20x SSC, pH 7.0
0.1% Tween-20
adjust pH to 6.5

**HYB-A**
1x HYB-B
0.05 mg/ml heparin
0.01 mg/ml yeast RNA
0.4 mg/ml sonicated salmon sperm DNA
Boil for 10 min and cool on ice for 3 min
store @ -20°C

**Blocking buffer**
1x PBS-T
1% BSA
2% Sheep serum

**AP staining buffer**
100 mM Tris pH 9.5
150 mM NaCl
10 mM MgCl₂
0.1% Tween-20

**Stop solution**
100 mM Tris pH 7.4
150 mM NaCl
10 mM MgCl₂
Appendix 2

Figure S1. Phylogenetic analysis of Loto and non-Loto clade genes. Green branches represent Loto clade genes. Black branches represent non-Loto clade genes. The blue branch with internal branches Ek-Loto and c399 fall between the Loto and non-Loto clades. The golden coloured branches indicate the incomplete sequences of c567 and c596 that fall between the Loto and non-Loto clades.